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Title: Serum Concentrations of Ergovaline/Ergot Alkaloids in Horses Grazing Endophyte Positive [E+] Pasture: A Preliminary Report

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Abstract. Ergot alkaloid toxicities such as tall fescue toxicosis from *Neotyphodium coenophialum*-endophyte-infected [E+] tall fescue pastures are important veterinary and economic problems. Tall fescue toxicosis apparently results from ingestion of vasoconstrictive ergot alkaloids produced by symbiotic fungal endophytes, and ergovaline is generally considered the critical toxin. To date, ELISA and HPLC with UV/fluorescence detection have been the predominant means of ergot alkaloid determination. These techniques, however, lack sufficient sensitivity and/or specificity to detect serum/plasma concentrations of specific ergot alkaloids. The present study reports the application of liquid-liquid extraction, HPLC and Electrospray(+) Ionization Mass Spectrometry (ESI-MS) with Multiple Reaction Monitoring (MRM) to detect specific ergot alkaloids in equine serum with a Limit of Detection estimated at 1 pg/ml. Estimated serum concentrations of ergovaline in horses grazing endophyte positive [E+] tall fescue pastures and showing clinical signs of toxicity ranged from (why <1.0?) <1.0 to 3.8 pg/ml, and its 8-positon epimer ergovalinine from (why <1.0?) <1.0 to 1.7 pg/ml, with concentrations of ergocryptine and its epimer, ergocryptinine, ranging from 1.2 to 6.8 pg/ml and 1.4 to 2.5 pg/ml, respectively. These part per trillion serum concentrations of ergot alkaloids are very low, but are consistent with estimated dose ranges and bioavailability for pasture ergot alkaloids. Additionally, the data suggest that these serum samples contain a number of ergot-related compounds, each of which may contribute to the overall clinical fescue toxicosis syndrome, and the specific identification of which will require broadening of the scope of this highly sensitive and specific analytical method.

Keywords: Horse; Ergovaline; Ergovalinine; Endophyte-positive; Fescue; Toxicosis.

1. Introduction

Ergot alkaloids have toxic properties which negatively impact equine reproduction, and cause the well described "fescue toxicosis" syndrome [1]. This syndrome is associated with the perennial grass *Festuca arundinacea*, or tall fescue, when infected with specific symbiotic fungal endophytes (*Neotyphodium coenophialum).* This grass, which spreads with seed propagation on over 35 million acres of pasture in the USA [2,3] is one of the most widely known forage grasses for horses. As a result of its widespread distribution, the toxic properties of infected "endophyte positive" or E+ pastures have considerable significance in equine reproductive processes [1]. Toxicity studies have described vasoconstrictive and uterine-stimulating properties of certain endophyte-produced alkaloids, including the ergotoxins [1]. Clinical signs of fescue toxicosis include gangrene, abortion, convulsions, suppression of lactation, hypersensitivity and ataxia.

Pregnant mares experiencing fescue toxicosis show adverse signs during the late gestation, including prolonged gestation, dystocia, agalactia, thickened placenta, poor foal viability and decreased plasma concentrations of prolactin and progesterone. Early pregnancy effects of fescue toxicosis have been investigated by Brendemuehl et al. at Auburn University [4] and include the following observations: 1) Do you mean ovarian? follicles during spring transition from January through April were lower in number and smaller in size in mares grazing on E+ fescue pastures compared to mares on E- pastures; 2) time to first ovulation was delayed from X days to X+ 43 days in mares ingesting E+ fescue; 3) cycling mares which were also affected by E+ fescue had prolonged luteal function, decreased pregnancy rates and increased embryonic death rates; 4) a smaller percentage (45%) of mares on E+ pastures were pregnant 14 days post-ovulation compared to 75% of mares grazed on E- fescue; 5) 30% of mares ingesting

the E+ fescue lost their pregnancies during early embryonic development compared to 8% of the mares on E- pastures.

Ergovaline [structure shown in Fig. 1] is considered the principal ergot alkaloid produced by *N. coenophialum*. In work related to that of Brendemuehl, Arns et al. [5] investigated the effects of ergovaline on establishment and maintenance of pregnancy in mares. Cycling mares (n=12) received diets that contained 0 ppb (NE), 150 ppb (LE), or 308 ppb (HE) what do NE, LE and HE stand for? ergovaline/kg diet in an orchard grass/grain concentrate mixture. Mares fed the HE diet tended to have a lower average daily weight gain compared to those mares fed NE or LE. Overall conception rates, cycles per conception, length of estrus and pre-ovulatory follicle size were similar in all three groups, but lower prolactin concentrations were found in LE and HE mares. The work suggested that dietary concentrations of ergovaline between 0 and 308 ppb/kg total diet did not produce adverse effects on reproductive performance in cycling mares. In contrast, comparison to several studies in the literature suggested to these authors that diets needed to exceed 325 ppb/kg total diet before signs of fescue toxicosis would be likely. This sentence should be rewritten!

Youngblood et al. [6] recently carried out studies on the effects of wild-type endophyte-infected tall fescue (E+) consumption by mares during the critical phases of placentation and fetal development in early pregnancy. Mares paired by gestational stage (days 65 to 100) were assigned to diets consisting of endophyte-free (E–) or E+ tall fescue seed (50% E– or E+ tall fescue seed, 45% sweet feed, and 10% molasses fed at 1.0% of body weight per day) for 10 days. Changes over time of serum progesterone concentrations relative to day 0 did not differ between groups. There was no negative pregnancy outcome, and ultrasonography indicated no increase in echogenic material in fetal fluids, normally thought to be possible warning signs of passage of

meconium, hemorrhage or accumulation of septic debris (6???). Apparent urinary ergot alkaloid concentrations as determined by ELISA were about 40 fold greater ($P < 0.01$) in mares consuming E+ compared with E– (532.12 ± 52.51 and 13.36 ± 2.67 ng/mg of creatinine, respectively what you mean by of creatinine?). Although no fetal loss was observed during the described study, elevated concentrations of urinary ergot alkaloid were consistent with depressed endogenous catecholamine activity as measured by reduced plasma concentrations of 3,4-dihydroxyphenylacetic acid, suggestive of an endocrine disruptive effect of hypothalamic origin.

Interpretation of these studies has been limited by our inability to accurately identify and quantify specific ergot alkaloids in the blood of horses grazing E+ positive pastures. However, successful use of highly sensitive LC-MS-MS for detection of low picogram/ml serum or plasma concentrations of medications used in racing horses [7] suggested application of these techniques in fescue toxicosis research [8,9]. In this communication we report 1) development of an electrospray(+)-tandem mass spectrometric method of sufficient sensitivity to enable evaluation of serum how about urine, later in the article you mentioned about urine collection ergovaline concentrations in horses on E+ pastures; 2) preliminary evaluation of ergovaline concentrations in serum samples collected from 4 horses grazing E+ pastures showing clear clinical signs of fescue toxicosis; and 3) apparent detection of other ergot alkaloid(s) in serum samples from these horses.

2. Materials and Methods

2.1 Horses

Quarter horse mares utilized in this study were maintained on pastures at the Mississippi Agriculture and Forestry Experiment Station, Mississippi State University [MSU] that were endophyte-positive, E+ (>90% contaminated), or negative, E– (<6% contaminated) for

approximately 10 weeks commencing mid-February 2004. Mares were fed while on pasture a daily ration formulated and fed to meet NRC recommendations for maintenance of late-term pregnant mares (NRC, 1989), and consisted of a commercial 10% C.P. sweet feed (All-Stock Sweet 10, Country Acres Feed Co., Brentwood, MO). Mineral blocks were provided along with fresh running water in pastures at all times. How did you come up with these numbers: The average monthly ergot alkaloid (ergovaline and ergovalinine) content typically ranged from 0.38 to 0.70 ppm with a mean of 0.52 ± 0.04 ppm for the growing season (March-June) for E+ pastures with peak concentrations observed during the months of April and May (0.8 to 0.9 ppm). On the other hand, the average monthly ergot alkaloid content of E- pastures ranged from undetectable to 0.18 ppm with a mean of 0.13 ± 0.02 ppm for the growing season (March-June) with peak concentrations of 0.20 to 0.34 ppm observed in individual samples (probably from E+ stands, ~ 6% of total stand in E- pasture) during the months of April and May. These values do not include other ergot alkaloid compounds present including the lysergic acids in endophyte-infecteted tall fescue forage with the fungus *Tymphimurium coenophialum*. All mares maintained on E+ pastures from March through mid-May demonstrated symptoms consistent with fescue toxicity.

2.2 Serum Samples from Fescue Grazing Horses:

. Blood samples were collected in March-April 2004 from mares 891, 516, 825 and 809 as mares exhibiting symptoms of fescue toxicosis of varying severity, including agalactia, placental thickening, problematic deliveries. Samples were collected using Vacutainer tubes (16x100 mm, 10 ml, Becton Dickson, Franklin Lakes, NJ); after collection, serum was obtained by centrifugation at 2000 rpm for 10 minutes, transferred to 13x100 mm glass tubes with caps and stored at –20° C. Volume of recovered serum ranged from 1.4 to 1.8 ml for the E+ horses, while

2 ml was the optimal volume for the method. Samples were also taken from horses on E- pasture and pooled for preparation of reference standards in E- serum matrix.

2.3 Urine sampling and ELISA analysis for ergot alkaloid

Urine Sampling and Urinary and Forage Ergot Alkaloid Analysis:

Urine was collected following cleaning of the perineal area with Betadine scrub solution (Medline, Mundelein, IL) and passing a sterile catheter into the urethra of the mare guided by a sterile rectal sleeved arm with sterile lubricant and collected in a sterile 50 ml vial. Urine samples, along with tall fescue forage samples, were analyzed for ergot alkaloid content by competitive ELISA using the ergot alkaloid monoclonal antibody 15F3.E5 that is specific to the lysergic moiety of all ergot alkaloids and expressed as ng/mg creatinine to correct for urine volume (Hill et al., 2000; Schnitzius et al., 2001). Ergot alkaloid content of forage (i.e., ergovalinine and ergovaline) was determined by Dr. L. P. Bush (University of Kentucky) using HPLC analysis as described previously (Craig et al., 1994; Schultz et al., 2006) and data expressed as total ergot alkaloid content of forage. The ELISA data of urine analysis provide total ergot alkaloids excreted in the urine, while the current study examines specific ergot alkaloids present in the plasma of the same mares.

2.4 Ergotoxin standards

Ergovaline was obtained as a tartrate salt from Dr. Miroslav Flieger of the Institute of Microbiology Academy of Sciences of the Czech Republic, Videnska, Prague, Czech Republic. Alpha-ergocryptine standard was obtained from Sigma Chemical, St. Louis, MO.

2.5 Solvents

HPLC-grade solvents included dichloromethane, methanol, acetonitrile and 93% formic acid from Fisher Scientific. Ultrapure water was obtained from a Milli-Q Water Purification System (Millipore, Billerica, MA).

Analytical Procedures:

2.6 Calibration Curves

Calibration curves were generated by spiking increasing amounts of stock solutions of ergovaline or ergocryptine into 2 ml of blank, i.e. E-, serum to give 0.01, 0.1, 1.0, 5.0 and 10.0 ng/ml concentrations. The "zero" sample comprised blank serum with no added ergovaline or ergocryptine. Each calibrator was prepared in duplicate, and one analytical injection was made from each. Resultant data were fitted to polynomial equations by application of Windows Excel XP software, and inverse plots enabled determination of concentrations in unknowns by simple substitution of area values, or summed values in the case of epimers, as described in Results. The r^2 for any given calibration curve was consistently greater than 0.99.

2.7 Liquid-Liquid Extraction

For ergovaline and ergocryptine extractions, 2 ml or the available volume of each serum sample were subjected to Liquid-Liquid Extraction (LLE) performed as follows. Two ml of each serum sample (including spiked serum samples utilized to generate the standard curve) were added to 15 ml screw-top glass tubes. Seven ml of dichloromethane (DCM) were added to each tube, which was then tightly capped. The samples were roto-racked for 15 min and then centrifuged at 4000 rpm for 15 min in a swinging bucket centrifuge (Beckman-Coulter Allegra 6R, Fullerton, CA) at room temperature. The aqueous layer was then removed and discarded along with the organic interface layer. The organic layer was transferred into glass tubes and evaporated to dryness under a stream of nitrogen in a 40° C water bath (Zymark TurboVap LV

evaporator, Hopkinton, MA). Residues were resuspended in 100ul of initial HPLC mobile phase (5% acetonitrile, 0.03% formic acid) then transferred to a microinjection vial and sealed.

2.8 MS-MS Tuning and HPLC

Samples were analyzed on a Varian 1200L tandem quadrupole LC/MS/MS (Palo Alto, CA) using a 20 µl injection volume. The mass spectrometer parameters were optimized while infusing 10 µg/ml of ergovaline dissolved in methanol:H_2O (10:90). Spectrometer conditions were as follows: ESI positive ion mode, drying gas temperature of N_2 300°C at 20 psi, N_2 nebulizing gas at 50 psi, needle voltage at 5000 V, shield voltage at 600 V, capillary voltage at +30 V, electron multiplier at 1700 V. Collision gas (argon) and collision energy were adjusted to obtain optimal molecular fragmentation for identification and quantification purposes.

Chromatography was performed on a Luna phenyl-hexyl column (30 mm x 1 mm, 3 µ particle size) equipped with a phenylpropyl guard column (4 mm x 2 mm) obtained from Phenomenex (Torrance, CA). Chromatographic conditions are described in Table 1.

2.9 Ergovaline & Ergocryptine Detection by LC/MS/MS:

Quantification of ergot alkaloids was based on the parent ion [M+H] and the most abundant daughter ion (designated as quantifier ion as determined by Varian Saturn MS Workstation version 6.40 software), while two additional fragments were used as qualifier ions to confirm identity. For ergovaline the [M+H] ion was m/z 534, quantifier ion m/z 268, and qualifier ions m/z 223 and 208. These were the same as previously reported for ergovaline [8]. Ergocryptine was handled similarly; here the [M+H] ion was m/z 576, the quantifier ion was m/z 305, and qualifiers were m/z 223 and 208. Ergovaline eluted between 8.5 to 9.5 min and ergocryptine eluted between 9 and 10 min Spectra of ergovaline and ergocryptine MRM mass spectra are shown in Fig. 2.

The limit of detection (LOD) using a signal-to-noise ratio minimum of 3, was estimated to be 10-fold below the lowest standard (10 pg/ml matrix, 2 pg on column), or ~ 1 pg/ml of matrix, 0.2 pg on column.

2.10 Molecular modeling

Ergopeptine three-dimensional structures were modeled using ACD/Chemsketch (Advanced Chemistry Development, Inc., Toronto, Ontario).

2.11 Steady-state pharmacokinetic projections:

Pharmacokinetic projections of the average daily intake of ergovaline required to produce the observed steady-state serum levels in these horses were based on the pharmacokinetic parameters defined for ergovaline by single I.V. dose as described by Bony et al. [10]. I am so confused about the following sentence, what are you trying to say? These projections for continuous oral consumption over time make the significant assumptions of 100% bioavailability, negligible effects for differences in horse strains used, and lack of interference by adsorption and storage of ergots in the fat compartment [Realini, et al, 2005]

3. Results

As set forth above, clinical signs of fescue toxicosis were observed in all animals grazing E+ pastures; for our preliminary analysis, we focused on four animals grazing E+ pastures that showed marked clinical symptoms of ergot alkaloid reproductive toxicity, with the goal of optimizing our likelihood of identifying toxicologically relevant serum concentrations of ergot alkaloids. Samples selected for analysis were from mares showing prolonged gestation, dystocia, agalactia and foal death, as set forth in table 1 , which lists days of gestation, physiological status of mares and foals, and serum ergot alkaloid concentrations. While the

gestational period of mare (#2) was normal, she delivered a dysmature foal, euthanized within 24 hours, and an edematous placenta with visible areas of surface villar necrosis. The remaining three mares delivered beyond their due date (4-18 days) two live and one still-born foal. The mare producing the still-born foal was euthanized six hours after delivery due to complications arising from severe dystocia. The foal from the other mare (#3) died 24 hours postpartum, with necropsy revealing meconium impaction and hemorrhagic enteritis. Parallel ergot alkaloid analysis, using an ELISA system [6], of urine samples corresponding to the date serum samples were drawn revealed urinary concentrations of apparent ergot alkaloid of 163.1, 236.9, 104.8 and 228.4 ng/mg creatinine is this typical way to express ergot alkaloid concentrations? for mares #1, #2, #3 and #4, respectively.

In our plasma analysis, ergovaline and ergocryptine were well separated by our HPLC conditions (Table 2) and a typical chromatogram of these two ergot alkaloid standards recovered from blank serum at 10 ng/ml is shown in Fig. 2. Duplicate standards containing a combination of ergovaline and ergocryptine in 2 ml blank serum with concentrations of 0.01, 0.1, 1.0, 5.0 and 10.0 ng/ml (without internal standard) were subjected to LC/MS/MS to construct standards curves based on quantification of MRM fragmentations (typical curves in Fig. 3).

In previous work [9], Lehner et al. determined that a useful correlation could be made between retention time and ergot molecular weight using various reverse phase HPLC chromatography conditions. The experimentally determined retention times of ergovaline and ergocryptine could therefore be used to extrapolate a linear relationship for direct estimation of the expected retention times of other ergotoxins, based on data from Lehner et al. using a similar HPLC column with different mobile phase, flow rate and temperature conditions. Table 2 lists these estimated retention time values along with those of the various ergotoxin epimers for which

retention times were established in the previous study. Epimerization of ergot alkaloids principally occurs by inversion of stereochemistry at the 8-position [11]; see Fig. 1.

We next directed our attention to the serum samples collected from selected horses grazing E+ pastures at MSU Based on the toxicological data, serum sample #1 was selected for evaluation by Single Ion Monitoring (SIM) for the M+H pseudomolecular ions of the principal potential ergotoxins: ergovaline (M+H = 534), ergocornine (562), ergocryptine (576), ergotamine (582), and ergocrystine (610) using the estimated retention times of Table 2. All ergotoxins were present along with a substantial matrix background signal, although only ergovaline and ergocryptine could be confidently identified due to unavailability of reference standards (Fig. 4).

The presence of a substantial matrix background signal during data analysis suggested the utilization of Multiple Reaction Monitoring [MRM] analysis to reducesignal from the serum matrix MRM. MRM analyses for ergovaline (m/z 534→268) and ergocryptine (m/z 576→305) are shown for serum sample #4 (Fig. 5); the background was lower and greater specificity was achieved-underscoring the advantages of the SIM approach. Specificity was less dependable, however, at these exceedingly low ergot concentrations, since measured values ranged above the method's LOD but below the concentration of the lowest calibrator in the standard curve. Fig. 6 shows that qualifier ions generally agreed with ergovaline and ergocryptine quantifier ion retention times; however, Table 3 shows that area ratios of qualifier ions to quantifiers were less dependable, owing principally to considerable background for these fragmentations at these low levels. Figs. 7 and 8 show, respectively, the paired peaks in all four samples for ergovaline and ergocryptine in comparison to the respective standards. Interpolation of summed areas on graphs such as that shown in Fig. 2 allowed estimation of the ergot alkaloid concentrations in samples

from each of the four horses, as presented in Table 5. Data from four different base samples taken in May of 2007 showed serum ergovaline/ergovalinine concentrations ranging from 1.5–5.5 pg/ml, generally agreeing with results presented in Table 5.

As indicated earlier, the paired peak phenomenon arises from epimerization of the ergot alkaloids, known to occur predominantly at the lysergic ring 8-position (Fig. 1, and [11]). Comparison of Figs. 7 and 8 shows that the relative ratio of ergovaline or ergocryptine to its presumed epimer present in each sample varied within the approximate ranges 1-4. Since at equilibrium in aqueous media epimers are not expected to be present at ratios differing substantially from 1.0 (0.92-1.5 for ergovaline, depending on pH; 0.89 ergocryptine [11]), this range 1-4 of epimer concentrations is consistent with and supports the assignment of these chromatographic peaks as epimers. The following sentence is too long and little confusing, should be rewritten: Additionally, this interpretation is based on the reasonable assumption that the ionization potential and energetics of fragmentation are equivalent for each epimer, consistent with the near identical appearance of the electron impact mass spectra for epimers ergonovine and ergonovinine, for example (cf. Wiley Registry mass spectral library, 7th edition, John Wiley & Sons, Inc.).

Table 5 supplies the ratio of each parent ergot to its epimer, based on raw areas; although the trends are broadly similar, ergocryptine appears to be somewhat less likely to epimerize than ergovaline under the conditions of this investigation, consistent with the findings of Smith and Shappell [11].

4. Discussion

The analytical method presented in this communication allowed highly sensitive detection of ergovaline and ergocryptine, their epimers, and apparently related ergot alkaloids in

the serum of horses grazing endophyte positive [E+] pastures that showed clearcut clinical signs of ergot alkaloid toxicity. In setting up the standard curve in equine serum, we selected 10 pg per ml of ergovaline and ergocryptine as our lowest concentration calibrators, and extended the concentration range to 10 ng per ml, our highest concentration calibrator. We chose this concentration range based on the pharmacokinetic data of Bony et al. [10], and also the pharmacological/toxicological range of activity for therapeutic drugs/toxins in serum or plasma, as set forth in the most recent edition of Goodman and Gilman [12]. The standard curves obtained were curvilinear and well fitted by a polynomial equation, with correlation coefficients, R^2, of at least 0.999. The Limit of Detection of the method was not determined, but is substantially less than the concentration of our lowest calibrator, and is estimated to be in the range of 1 pg/ml. Such sensitivity should allow for detection of ergot alkaloids including presumably ergovaline in serum samples from horses grazing E+ pastures.

Using this analytical method, ergovaline was detectable in the serum of all four horses grazing E+ fields (Fig. 7, Tables 4/5) at concentrations ranging from 0.7 to 3.8 pg/ml, below the concentration of our lowest calibrator. However, ergovaline was not necessarily the principal ergot alkaloid present; preliminary evaluation of these data suggest that ergocryptine and other ergot-related compounds are also present, and the specific identification of these companion ergot alkaloids will require further analytical work supported by access to the required authentic reference standards.

A second significant finding was that ergovaline and ergocryptine were apparently also present in these serum samples as their epimers. Epimerization occurs when an asymmetric carbon atom, in this case most likely carbon atom #8, undergoes inversion of orientation, yielding an epimeric form of the substance. Because our ergovaline and ergocryptine standards

1 did not epimerize significantly during the analytical procedure, these epimers were presumably
2 present *in vivo* in the serum samples of the horses in question. The presence of these epimers
3 had the effect of reducing the analytical concentration of each ergot alkaloid expressed as total
4 ergot alkaloid, ergovaline and ergocryptine, respectively, by between 50 and 33%..
5 Epimerization is a characteristic of the stereochemistry of a number of ergot alkaloids,
6 and Fig. 1 (top schematic) illustrates that carbons 8, 11, 2', 5', 11' and 12' of the basic ergotoxin
7 skeleton are asymmetric carbon atoms, imparting stereochemical properties to these alkaloids.
8 Dehydration at carbons 11' and 12' removes the asymmetric properties of these carbons, and
9 forms the basis of certain known and hypothesized ergots (Table 7 in Lehner, et al. [9]). The
10 stereochemistry of carbon 8 in Fig. 1 is also subject to alteration, in this case by inversion of
11 orientation, the process called epimerization. Given the striking differences in 3-d structure seen
12 in Hyperchem-modeled precursor and product (Fig. 1 A & B), epimerization at a position such as
13 carbon 8 may be expected to affect the chromatographic retention time of the affected substance,
14 and also to alter its fragmentation pattern. While the effect of epimerization on toxicity has not
15 been established, epimer transformation has been generally considered to eliminate biological
16 activity [13].
17 Earlier work by Lehner et al. [9] identified a series of major ergot alkaloid peaks by LC-
18 MS-MS, including proposed epimer retention times. Similar to data reported for ergovaline
19 under these reverse phase chromatographic conditions, we found its epimer ergovalinine nearly
20 overlapped with that of the parent compound, (8.67 min and 8.94 min, Fig. 5) results reported
21 here. Lehner et al. [9] did not identify a reasonable ergocryptine epimer candidate, listing 7.15
22 min as the retention time of the major epimer and 7.4 as hypothetical minor epimer, eluting just
23 after ergovaline. Those results were similar to the results reported in the present work, with the

ergot standards at 8.7 min and 9.3 min for ergovaline and ergocryptine, respectively, yet the ergocryptine data presented here show a consistent pairing of peaks at retention times 8.055 min and 9.282 min. The most reasonable interpretation is that this second peak in the current data is the ergocryptine epimer ergocryptinine.

In comparing our current data with our previously reported data, it should be remembered that samples sources differ. Structures reported by Lehner et al. [9] were from analysis of grass extracts from Oregon pastures, while our current analyses were on equine serum from horses grazing on endophyte-infected tall fescue pastures in Mississippi. In this regard, stomach acidity and elevated temperature chemistry might easily cause epimerization, apparently in some ergot alkaloids more than in others. In any case, it appears most simply that the ergovaline 6.49-6.53 min peaks [9] have occurred as 8.67-8.69 min peaks in this chromatographic system whereas ergocryptine 7.15-7.40 peaks have given rise to 9.28-8.10 min peaks in this system.

We can readily estimate the daily dose of ergovaline required to produce these relatively low [5 pg per milliliter or less] serum concentrations of ergovaline using Bony's previously reported pharmacokinetics data for ergovaline in the horse [10]. Assuming that the systemic clearance of ergovaline in the horses included in the present study is similar to that estimated by Bony et al. (Cls= 1200 ml/hr/kg), pharmacokinetic modeling suggests that in order to obtain a 5 pg/ml average steady state concentration of ergovaline, a horse would need to have ergovaline enter its blood stream at 6 ng/kg/hr, or 144 ng/kg/24 hour period of ergovaline, or 65 μg/day for an average 450 kg horse. Information on the oral bioavailability of ergovaline in the horse is not accessible, but review of the literature suggests that its bioavailability is likely to be low; for example, the oral bioavailability of ergotamine is considered to be on the order of 2-5% [18,20].

If we assume an oral bioavailability of 5% for ergovaline, then the average daily oral dose of ergovaline needed to produce the indicated serum concentrations of ergovaline will be 120 ng/kg/hr exposure, or exposure to 1.296 mg per day/horse of ergovaline, in good general agreement with field estimates of ergovaline exposure from E+ pastures associated with clinical signs of fescue toxicosis [L. Lawrence et al., 2007 personal communication].

We note that these serum concentrations of ergovaline are in the order of a thousand fold less than the nanogram per milliliter plasma concentrations of ergovaline reported by Bony et al. [10], who also reported observing clinical signs consistent with ergot alkaloid toxicosis in their horses. What is unclear from the Bony report is for how long these clinical signs were observed after the intravenous administration of ergovaline, since the point of disappearance of the clinical signs of ergovaline toxicity would suggest a "No Effect" plasma level for ergovaline, at least for ergovaline administered intravenously to horses.

Another consideration regarding this work is that there remains a possibility that the choice of serum as the matrix for examination may have provided artificially reduced ergot concentrations. Although detailed pharmacokinetic data on ergot alkaloids in large animals is, with the exception of work done by Bony, et al. [10], sparse, information developed for the semi-synthetic ergopeptine-derived drugs dihydroergotamine [21-22] and bromocriptine [23-24], for example, indicate that these compounds tend to be highly protein bound, ~93% and 90-96% respectively. In this regard, we suggest focusing future work of this type on plasma collection in preference to serum in order to optimize our ability to accurately quantify these very low total blood ergot alkaloid concentrations.

In closing, the conclusions from this preliminary analysis seem inescapable; if ergovaline is indeed the active agent in fescue toxicosis, it is an unusually potent plant toxin, active at the

low picogram per milliliter serum levels, on the order of 10 times more potent than scopolamine; alternatively, ergovaline may be a precursor toxin or pro-toxin, and fescue toxicosis may be due to a metabolically transformed product of ergovaline, or, and perhaps more likely, fescue toxicosis may be due to ergovaline in combination with another member or members of the cohort of ergot alkaloid toxins, acting in combination.

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Table 1. Mare sample collection information and ergot alkaloid toxicity/pregnancy outcome:

Mare ID	Date sample collected	Sample #	Serum volume available (ml)	Day gestation sample collected	Date mare due (~340 days)	Day gestation delivered	Pregnancy outcome
819	4/01/04	1	1.8	341	3/31/04	351	Dystocia, foal died at birth
516	3/25/04	2	1.7	332	4/2/04	336	Foal euthanized 24 h postpartum
825	4/01/04	3	1.7	317	4/23/04	365	Foal died 24 h postpartum, meconium impaction
809	4/01/04	4	1.4	300	5/11/04	339	Viable foal

Table 2. Conditions for HPLC during LC/MS/MS.

Time during gradient (min: sec)	**Mobile phase composition**	
	%A[1]	**%B[2]**
0:00	100	0
3:00	100	0
15:00	10	90
18:00	10	90
19:00	100	0
30:00	100	0

[1]Solvent A = 95% H_2O + 0.03% Formic Acid + 5% Acetonitrile
[2]Solvent B = Acetonitrile + 0.03% Formic Acid
Flow rate = 0.15 ml/min throughout
Collision gas was set at 2 mTorr and collision energy was set at –20 V

Table 3. Predicted HPLC retention times for ergotoxins and their 8-position epimers. based on Lehner, et al. [9] by fit to the equation y = 0.9251x + 2.7094, in turn based on retention times for ergovaline (8.7133 min ± 0.0263) and ergocryptine (9.3239 min ± 0.0335), n=10.

Compound	RT (2005) Luna HPLC	RT current [a] or predicted [b]	mw
Ergonovine	5.56	7.853	325
Ergonovine epimer	5.79	8.066	325
Ergovaline	6.49	***8.713***[a]	533
Ergovaline epimer	6.53	8.750	533
Ergocryptine	7.15	***9.324***[a]	575
Ergocornine	6.9	9.093	561
Ergocornine epimer	6.8	9.000	561
Ergotamine	**6.85**	8.898	581
Ergotamine epimer	**6.96**	8.982	581
Ergocrystine	7.37	9.527	609
Ergocrystine epimer	7.1	9.278	609

[a] As determined empirically by authors using standards in horse serum, and used to adapt Lehner et al data to current chromatographic conditions. Retentions time for ergovaline was 8.7133 min ± 0.0263, and 9.3239 min ± 0.0335 for ergocryptine (n=10).

[b] Predicted from equation derived for retention times of ergot compounds based on molecular mass y = 0.9251x + 2.7094 [9], adjusted for observed retention times of ergovaline and ergocryptine.

Table 4. Relative abundance of qualifier ion area to quantifier ion area for the principal stereoisomer of ergovaline and ergocryptine in horse serum samples compared with those for standards in serum. Expected ranges are based on Association of Official Racing Chemist (Lexington, KY) criteria of ±20% absolute or ±40% relative abundance.

Compound	Fragmentation	Samples		Expected ranges for R.A., %		Standards (10,1000, 5000 pg/ml)	
		Relative abundance %, average (n=4)	std dev, %	Min, %	Max, %	Relative abundance %, average (n=3)	std dev
Ergovaline	534.0 > 268.0	100.0	0.0			100.0	0.0
	534.0 > 223.0	104.1	31.9	38.3	89.3	63.8	1.9
	534.0 > 208.0	22.3	15.6	4.8	44.8	24.8	7.8
Ergocryptine	576.0 > 305.0	100.0	0.0			100.0	0.0
	576.0 > 223.0	140.6	87.7	52.8	123.1	87.9	8.1
	576.0 > 208.0	15.8	8.4	10.4	50.4	30.4	3.9

Table 5. Calculated serum ergovaline (A) and ergocryptine (B) concentrations, estimated epimer concentrations and peak area ratios. (pg/ml, $\bar{x}$ = mean)

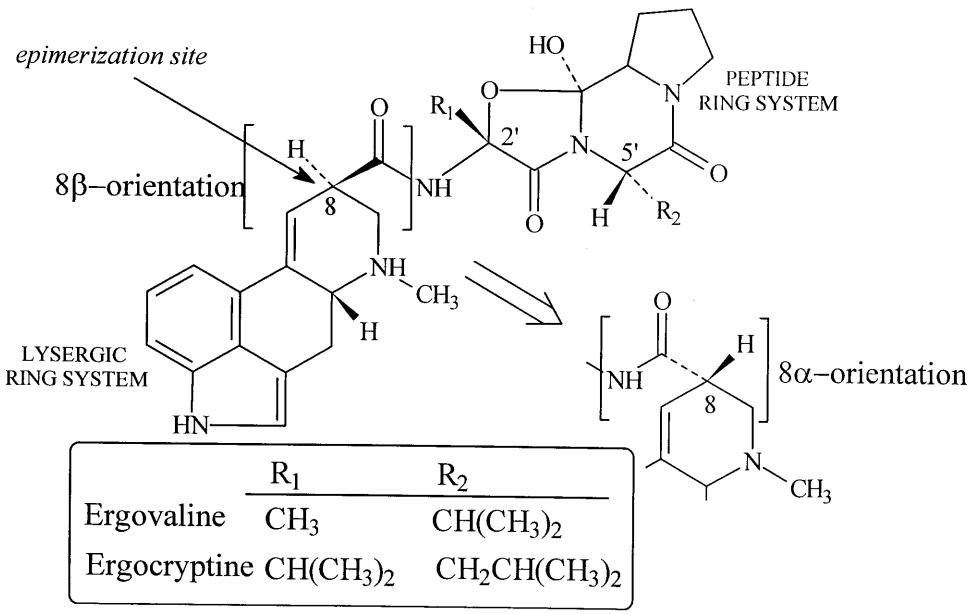
A

Sample #	ergovaline area	epimer area	Ergovaline/epimer area ratio	ergovaline pg/ml	epimer pg/ml	total pg/ml
1	20579	20721	0.99	0.7	0.7	1.5
2	30808	33326	0.92	1.2	1.4	2.6
3	86666	39845	2.18	3.8	1.7	5.5
4	51492	21986	2.34	2.7	1.0	3.7
$\bar{x}$	47386.3	28969.5		2.1	1.2	3.3

B

Sample #	ergocryptine area	epimer area	Ergocryptine/epimer area ratio	ergocryptine pg/ml	epimer pg/ml	total pg/ml
1	37511	33411	1.12	1.2	1.4	2.2
2	40388	54124	0.75	1.4	2.5	3.2
3	185651	41822	4.44	6.8	1.3	8.2
4	121822	50258	2.42	4.4	1.7	7.5
$\bar{x}$	96343	44903.8		3.5	1.7	5.3

Figures:



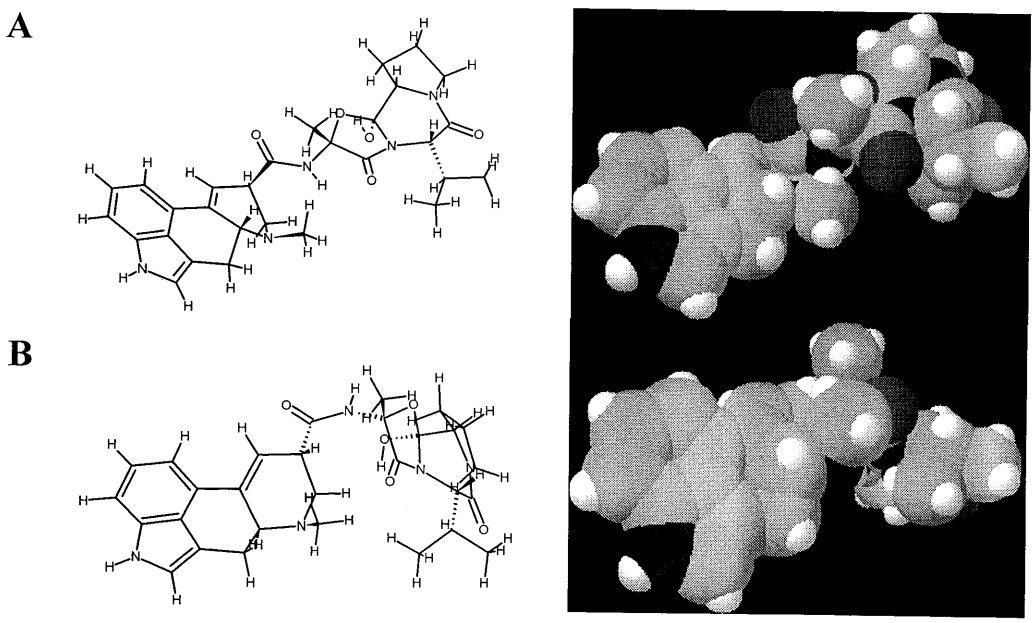


Figure 1. (legend on next page)

Figure 1. Top: Ergopeptine structure, with differences between ergovaline and ergocryptine existing in the alkyl substituents of the peptide ring system designated R_1 and R_2. . The epimerization site at position 8 of the lysergic ring system results in transformation of ergovaline and ergocryptine to ergovalinine and ergocryptinine, respectively.

Stick figure and space-filling representations of ergovaline (A) and its 8-α epimer ergovalinine (B), the lysergic ring system to the left.

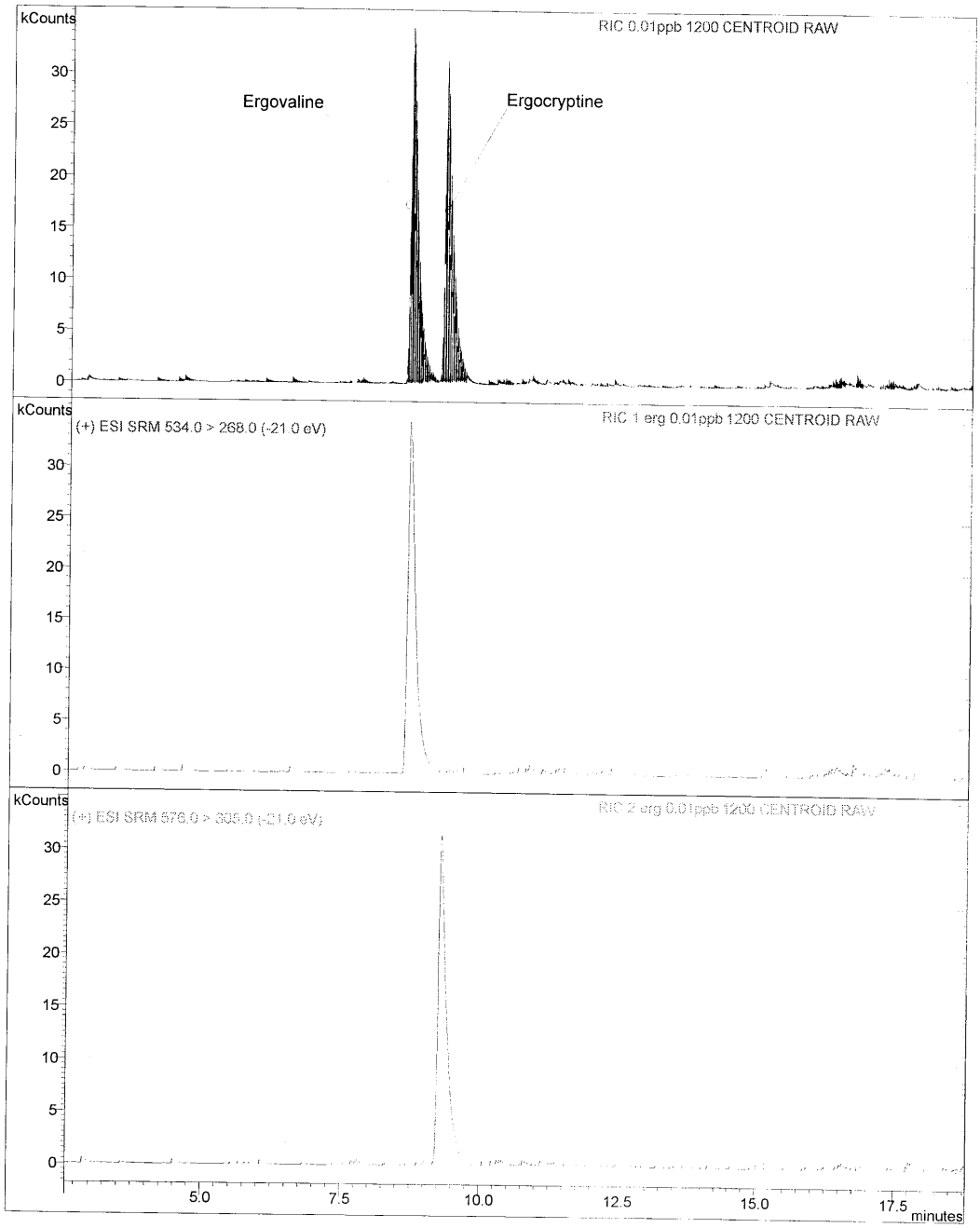


Figure 2. Chromatogram of ergovaline and ergocryptine standards in serum. Top: 0.01 ppb ergovaline and ergocryptine standards were run by the full SIM method, with resultant retention times of 8.698 min (ergovaline) and 9.308 min (ergocryptine). Middle & bottom: ion chromatograms for m/z 534→ 268 (middle) and 576→ 305 (bottom).

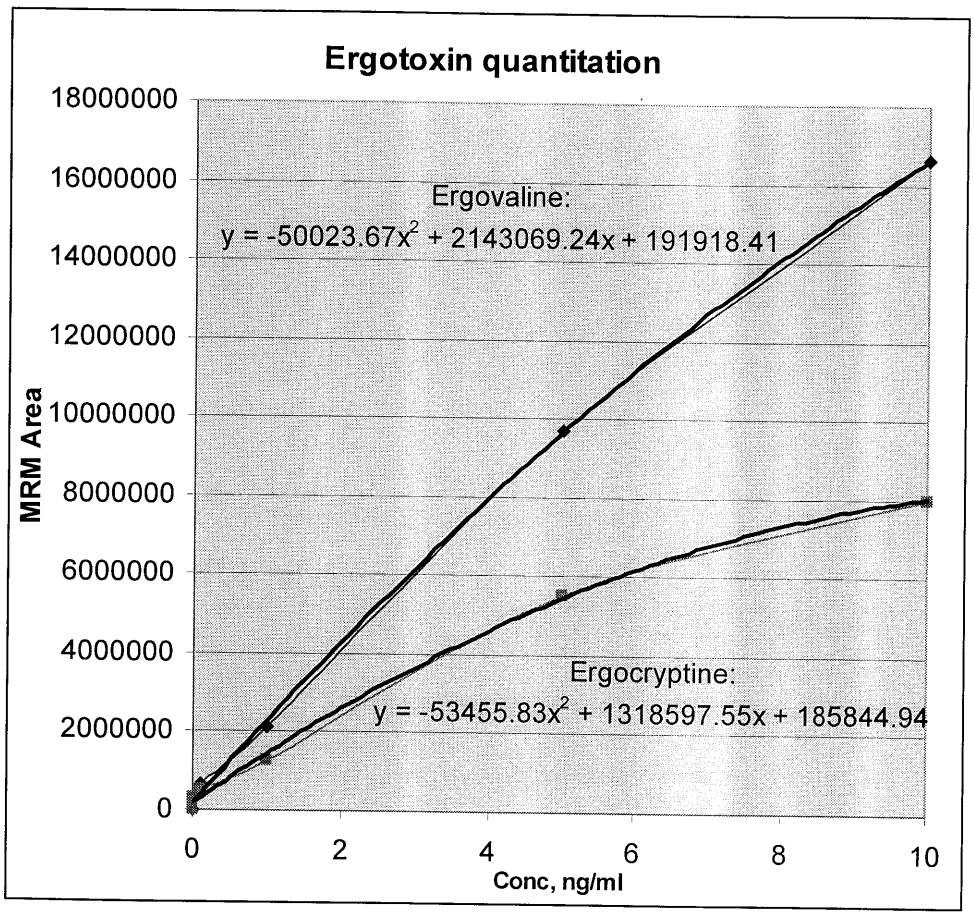


Figure 3. Standard curve generated for ergovaline and ergocryptine. Mean of duplicate injections plotted. The heavy trend lines indicate fit to the polynomial equations displayed on the graph. Ergovaline concentrations estimated by fit to the inverse plot as $y = 1.234526E\text{-}14x^2 + 3.968044E\text{-}07x$, where y=concentration in ng/ml and x = MRM peak area; similarly, ergocryptine concentrations estimated by fit to the inverse plot as $y = 1.133700E\text{-}13\ x^2 + 3.270618E\text{-}07x$.

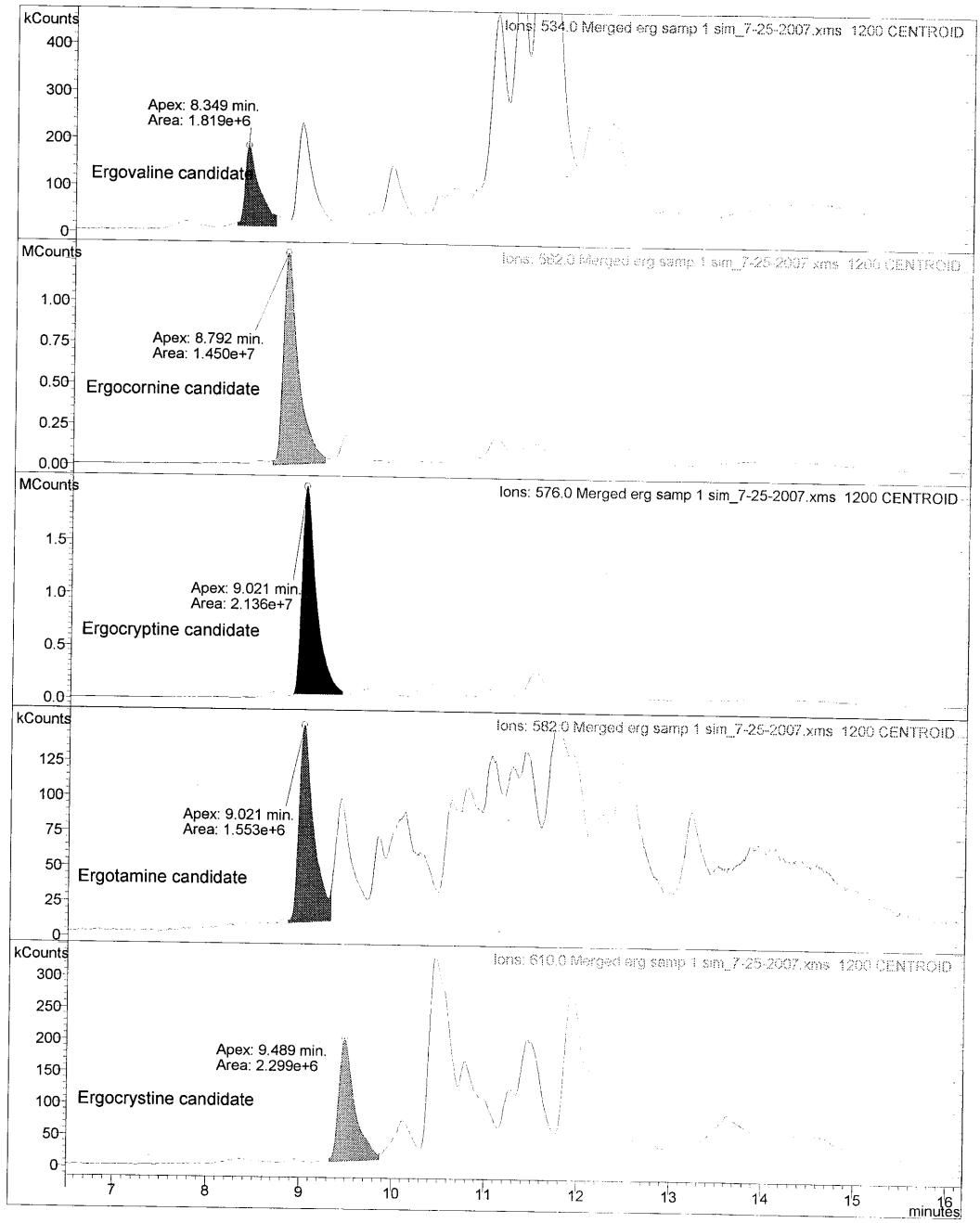


Figure 4. Individual ion chromatograms for horse serum sample #1, examining the ergot-specific M+H m/z values of 534, 562, 576, 582, and 610 (top to bottom). Peak identities (shaded areas) were proposed based M+H m/z and by reference to predicted retention times as in Table 2 of known ergot compounds.

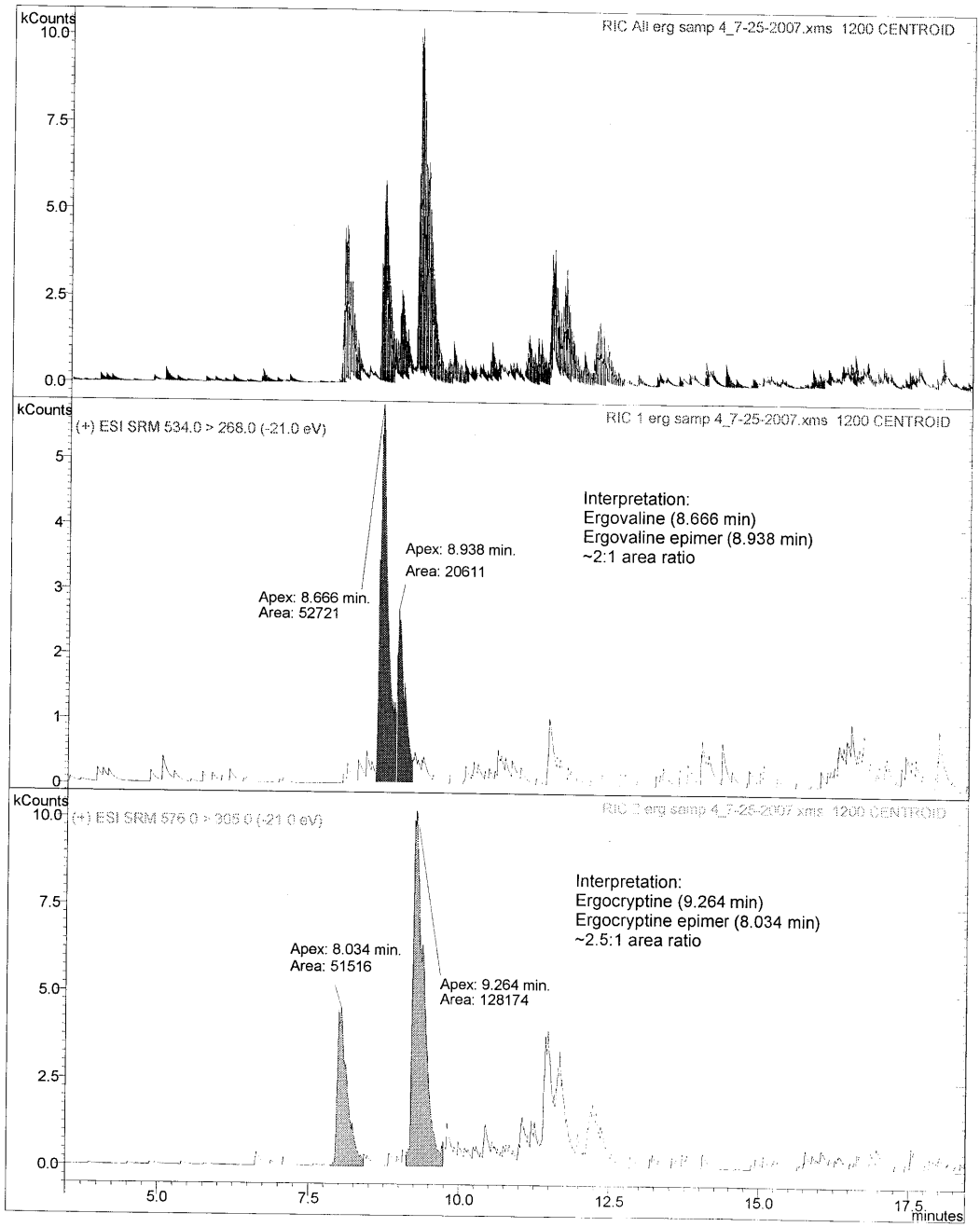


Figure 5. Full MRM data for horse serum sample #4 (top) showing presence of ergot-related material, particularly for the m/z 534→208 ergovaline (middle) and m/z 576→305 ergocryptine (bottom) chromatographic traces, with peaks of interest highlighted.

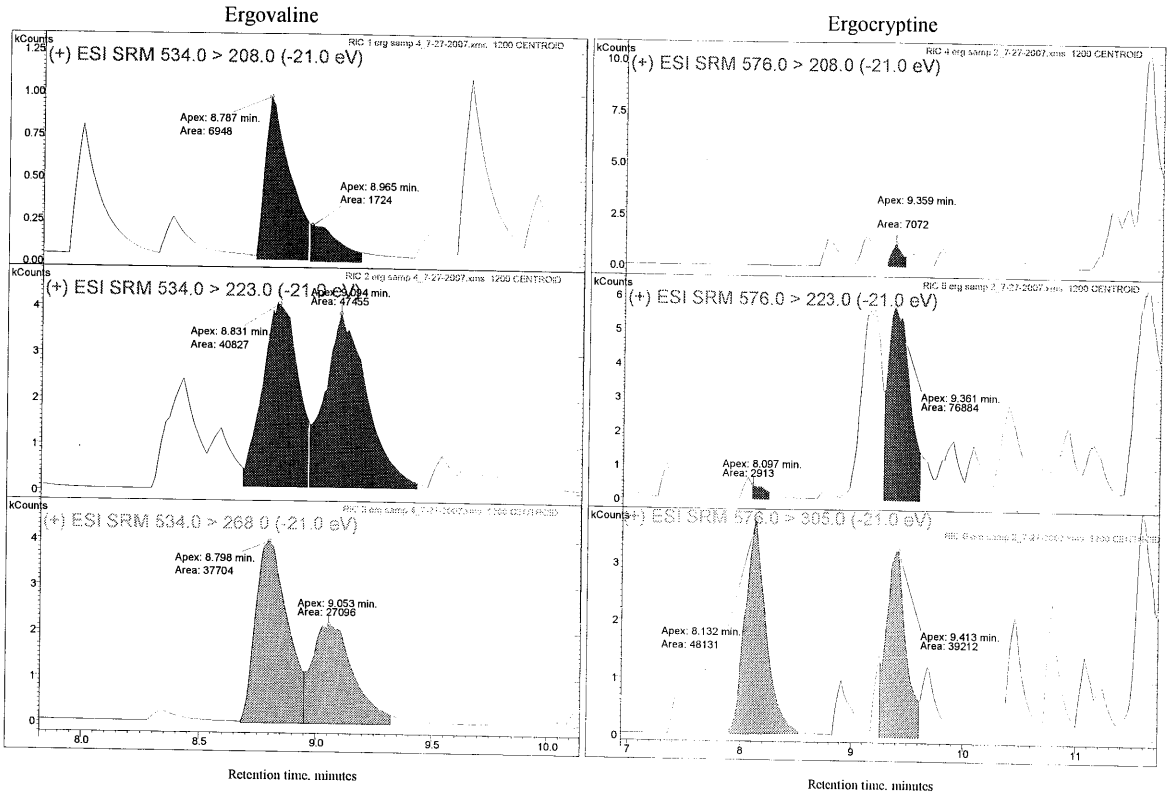


Figure 6. Determination of specificity in ergovaline (left) and ergocryptine (right) identifications, as shown for sample 4. Qualifier fragmentations for ergovaline at 8.8 min retention time included m/z 534→223 & 208, and for ergocryptine at 9.4 min included m/z 576→223 & 208. **Proposed** alpha-epimers at 9.0 min for ergovaline and 8.1 min for ergocryptine show quantitative differences in fragment yields. s.

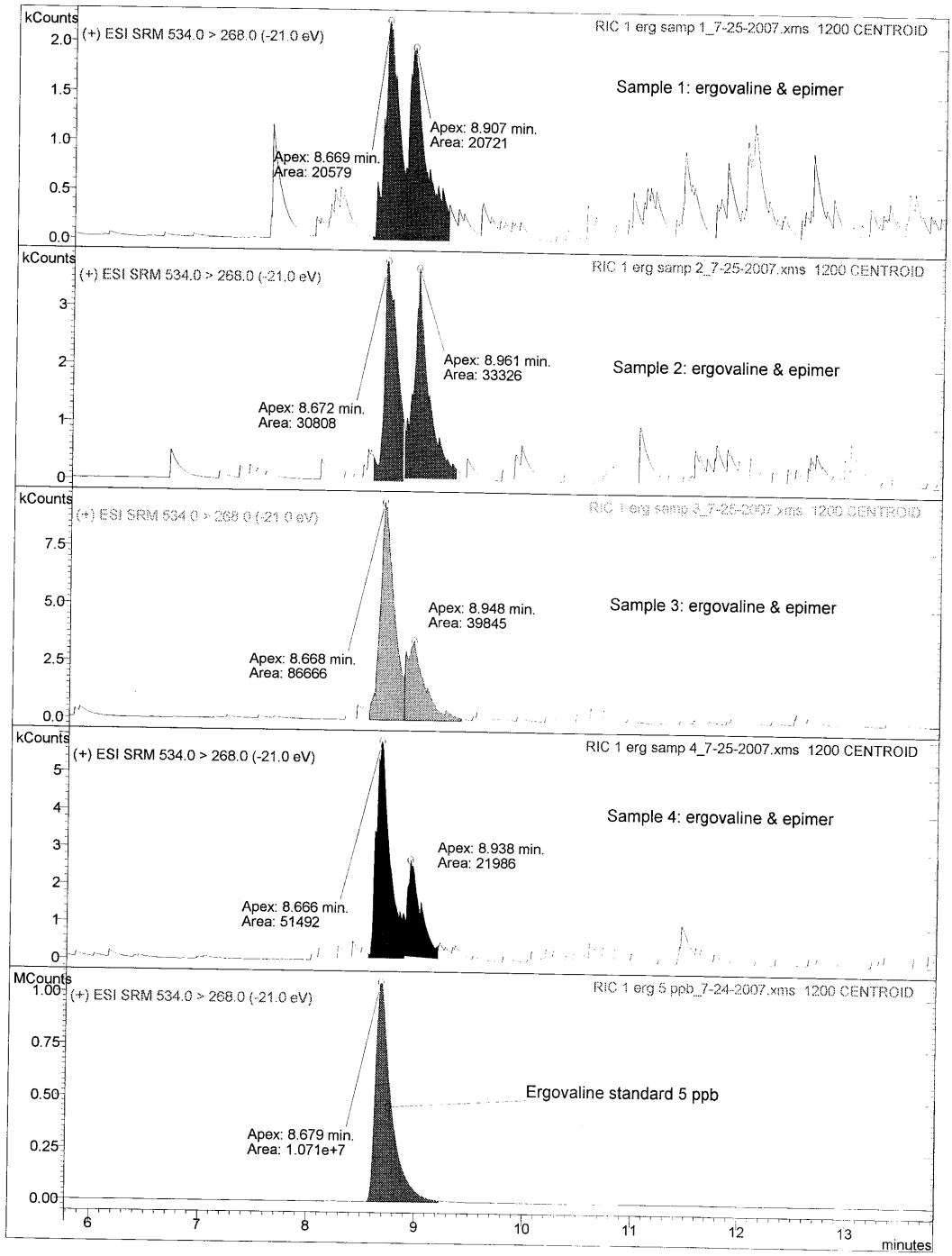


Figure 7. Survey of m/z 534→268 ergovaline peaks in samples #1-4 (from top down) in comparison to ergovaline standard (bottom). Peaks for ergovaline and its epimer are shaded.

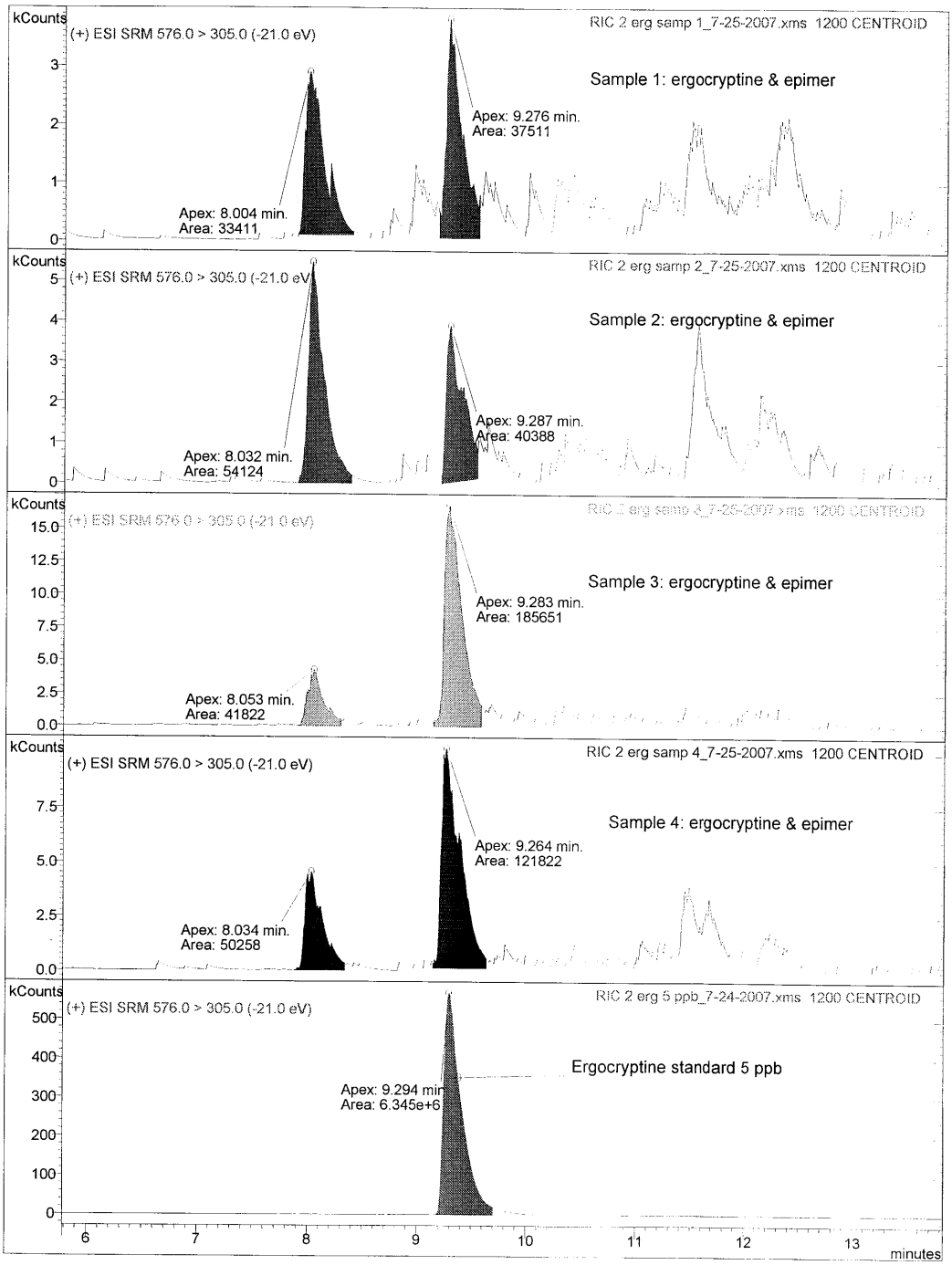


Figure 8. Survey of m/z 576→305 ergocryptine peaks in samples #1-4 (from top down) in comparison to ergocryptine standard (bottom). Peaks for ergocryptine and its epimer are shaded.

Additional figures:

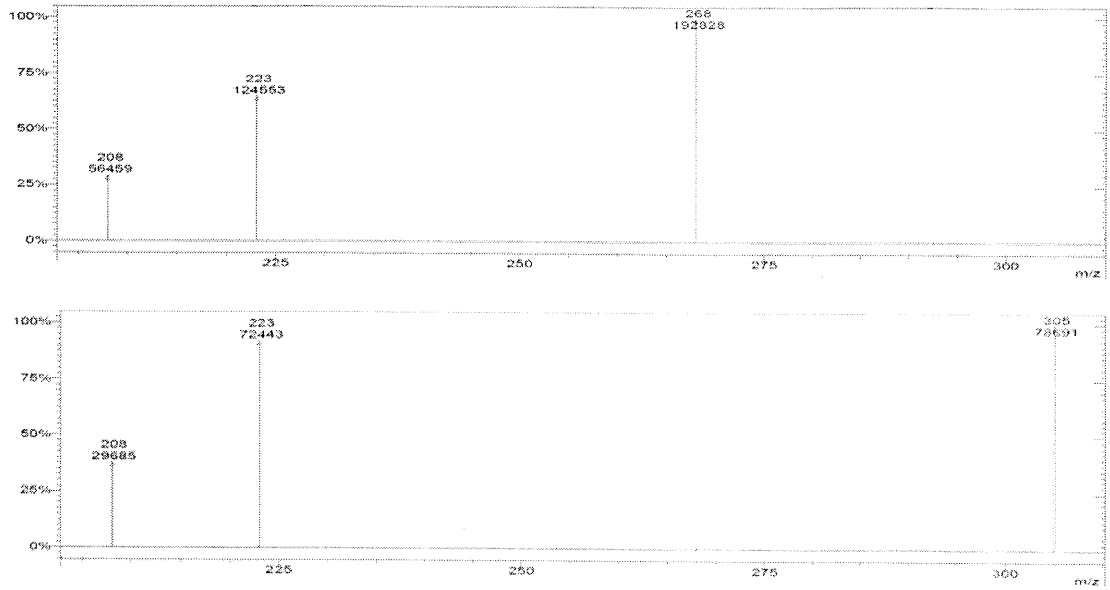


Figure. Mass spectra for ergovaline [Top] and ergocryptine [Bottom] standards spiked in serum, extracted and chromatographed and run by the full SIM method. Top: 5 ppb ergovaline 8.743 minute peak. Bottom: 5 ppb ergocryptine 9.355 minute peak.